Molecular cloning and analysis of the partial sequence of *Rhinopithecus roxellanae* growth hormone gene[1]

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Abstract Growth hormone gene (*GH*) of *Rhinopithecus roxellanae* was amplified by PCR based on the sequences of the reported mammalian growth hormone gene for the first time. The amplified fragment was about 1.8 kb. It was cloned and its upper stream was sequenced. This sequencing region consists of a 5′flanking regulatory region, exon I and part of exon II, intron I of growth hormone gene. Comparing the corresponding sequences of growth hormone gene between *Rhinopithecus roxellanae* and the porcine, we concluded that the homology reached 81% in the region, and there was high conservation in the 5′flanking sequence. The kinds of amino acids of exon I and exon II for about 90% were the same to those in pig. Many mutations occurred in the degenerate site of the triplet code. In the nucleotides of intron I, there were only 72% homologies with those in pig. It means that introns and 3′flanking sequence maybe play an important part in growth hormone gene regulation of the different animals.

Key words: *Rhinopithecus roxellanae*, Growth hormone gene, PCR, 5′flanking regulation region, Exon, Intron

Introduction

Growth hormone gene (*GH*) of the vertebrate, a 22 kDa protein hormone synthesized in anterior pituitary gland, was required for preadult growth (Franchimont and Burger 1975; Barinaga *et al.* 1985). The genes encoding rat, human, bovine, porcine, ovine, turkey and duck *GH* have been well studied at both protein and gene levels (Page *et al.* 1981; DeNoto *et al.* 1981; Woychik *et al.* 1982; Vize *et al.* 1987; Byrne *et al.* 1987; Foster *et al.* 1990). Growth hormone gene is also a member of a multi-gene family. Amino acid and nucleotide sequencing data suggest that the gene for growth hormone, prolactin (PRL) and chorionic somatomammotropin (CHS) all arose from duplications of a single primordial gene sequence. Analysis of this gene family in the human revealed that, in addition to the prolactin and chorionic somatomammotropin genes, there are at least two growth hormone-related genes. The rat genome, however, appears to have only a single growth hormone gene. Further analysis involving the evolution of this gene family awaits the characterization of the growth hormone sequences in several other species.

In this paper we described the isolation and partial nucleotide sequencing of the *Rhinopithecus roxellanae* growth hormone gene. The gene was approximately 1.8 kb in length. According to our results and the nucleotide sequences reported for the porcine growth hormone gene, we compared the growth hormone gene sequences from two different animals.

Materials and methods

The *Rhinopithecus roxellanae* muscle tissue was presented for the experiment by Dr. Li Ming in Institute of Zoology, the Chinese Academy Sciences. The PCR kit and all restriction enzymes were purchased from Promega company. *E.coli*JM109 strains and pUC19 vectors were purchased from Sino-American Biotechnology Company.

The genomic DNA extraction process from *Rhinopithecus roxellanae* muscle tissue

The muscle tissue samples suspended in PBS are crushed into small pieces using the plunger from 3 mL syringe, the pieces are then passed 10 times through a 3 mL syringe to disperse the sample into small fragments and single cells. The best DNA yields require small pieces of tissue, and we have found that the use of a 3 mL syringe as described provides a high yield of small fragments suitable for digestion. After allowing large debris to settle for 1

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min, the cell suspension was transferred to a 5 mL culture tube and centrifuged at 1 200r.p.m. (300 g) for 7 min at 4°C. The cell pellet was resuspended in lysis buffer [400 mM NaCl, 100 mM Tris-HCl (pH 8.5), 5 mM EDTA, 0.2% SDS, 20 μg/mL RNase A and 500 μg/mL proteinase K], and 3 mL of each lysate was placed in a 10 cm culture dish or 1 mL of lysate to a 6 cm culture dish. Different tissues require varying quantities of lysis buffer. We lyse 0.3 g muscle in 3 mL of lysis buffer. The plates are incubated overnight at 37 °C. After disgestion, an equal volume of isopropanol was added to each plate and mixed by gentle rocking. The dishes were left undisturbed at room temperature at least 2h until the white DNA precipitate can be seen at the bottom of the dish. Because the DNA precipitate adheres to the plastic dishes, the supernatant can be poured off gently, and the DNA was washed twice with ice cold 70% ethanol. After washing, the DNA was air dried briefly (10 min), and then dissolved in an appropriate amount of TE buffer by incubation at 37 °C for 2h or at 4 °C overnight. Because of the minimal manipulation of DNA during the isolation procedure, almost all the DNA was high molecular weight (Wu *et al.* 1995).

PCR amplification reaction

PCR conditions referred to the PCR kit directions. The reaction process was made by Perkin Elmer DNA Thermal Cycler960.

The cloning of PCR amplification products and identification

The PCR amplification products were recovered using the method of agarose gel electrophoresis of low melting point. The solution was then extracted twice with Tris-saturated phenol, followed by three chloroform extractions. After precipitating the DNA with ethanol, the sample was allowed to fill the end of amplification fragments in 24 μL PCR products, 5μL Klenow enzyme buffer (×10), 1.6 μL dNTP (2.5 M), 0.5 μL Klenow enzyme (10 U/μL), 18.9 μL redistilled water.

The growth hormone gene fragments (1.8 kb in length), which were amplified by PCR and filled the end, were ligated into the *Hinc*II sites of the cloning vectors pUC19 to form the recombinant plasmids pUC19-GH, transformed into *E.coli* JM109 cells, and transformants were selected on LB-Amp agar containing IPTG and X-Gal. This assay detects impurities, which affect ligation and transformation efficiencies as well as the presence of contaminating exonucleases that would destroy the *β*–galactosidase reading frame, resulting in white colonies. After selecting the white colonies, the sample was allowed to extract the plasmid DNA. The purified DNA was digested with both *Eco*RI and *Hind*III for the identification.

The partial growth hormone gene sequencing and analysis

The purified recombinant plasmid pUC19-*GH* was sequenced using ABI PRISM™377 DNA sequencer by Bao Biotechnology Company. The sequencing result was analysed with DNASIS software.

Results and discussion

The PCR amplification and cloning of *Rhinopithecus roxellanae GH*

We designed a pair of primers based on the sequences of the reported human, rat, bovine, porcine and ovine growth hormone gene.

The upper primer: 5′-GAGGAGCTTCTAAATTATCCATTAGCACA-3′.

The lower primer: 5′-CCC/TA/GGC/GAACTAGAAGG/CCACAGCTGG/CC/TC/TTC-3′.

The upper primer is located about 180 bp upstream from cap site, while the lower primer is located in the end of exon V and 3′flanking sequence. The *Rhinopithecus roxellanae* growth hormone gene was amplified by PCR, taking the genomic DNA as template. The products of PCR were detected using 0.8% agarose gel electrophoresis. The result was shown in Fig. 1. There is a specific banding localized on 1.8 kb place.

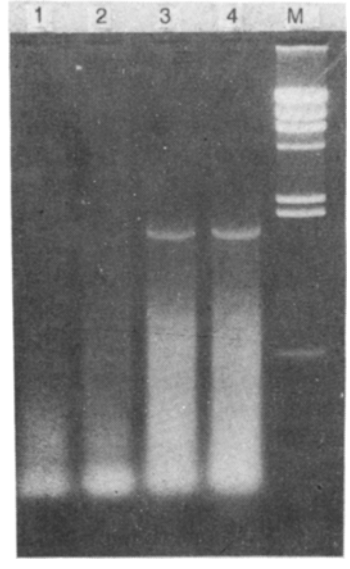

Fig. 1. The PCR products corresponding to the growth hormone gene of *Rhinopithecus roxellanae*

M: DNA/*Hind*III marker; 1-2: PCR products; 3-4: Negative control

The purified pUC19-*GH* DNA, which was extracted by the transformants, was digested with both *Eco*RI and *Hind*III. The result is shown in Fig. 2. There are

about 2.7 kb and 1.8 kb banding in the first path. This result is tally with the theory.

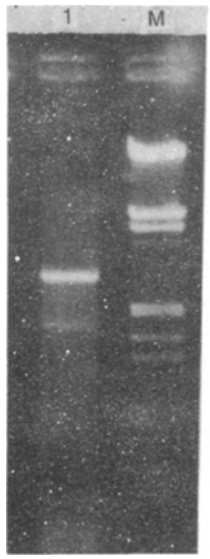

Fig. 2. Identification of pUC19-*GH* by *Hind*III and *Eco*RI digestion

M: DNA/*Hind*III and *EcoR*I marker;
1:pUC19-*GH* by the double enzyme digestion

The partial sequence and analysis of growth hormone gene

The identified recombination plasmid made sequencing material. 5'flanking sequence, exon I, intron I and partial exon II nucleotides of growth hormone gene were sequenced, which is 585 bp in length. Comparing the corresponding sequences of growth hormone gene between *Rhinopithecus roxellanae* and the porcine (Vize 1987), the result was shown in Fig. 3.

While growth hormone genes of only sequencing part of *Rhinopithecus roxellanae* and the porcine were compared with each other, the incorresponding nucleotides were indicated by ":" because of frameshift mutation. This revised comparison involved inserting spaces to allow a more accurate place of corresponding sequence. In growth hormone gene the studied portion contains 591 nucleotide sites. Comparison of the *GH* from the porcine (DeNoto *et al.* 1981) reveals that *Rhinopithecus roxellanae GH* shares 81% sequence homology with *GH* of porcine.90% homology is found also when the sequenced exon region of *Rhinopithecus roxellanae GH* is compared with that of porcine. Many mutations occurred in the degenerate site of the triplet codes. In the nucleotides of intron I, there were only 72% homologies with those in the pig. Through analyzing the sequence by the DNASIS software, it is found following restriction enzyme sites: *Dra* II (115), *BamH* I (137), *Dra* II (170), *Pst* I (208), *BstX* I (342), *Sma* I (373), *Dra* II (444) and *Apa* I (581).

A sequence related to TATAAA is found approximately 23 base pairs upstream from the start of transcription. The related sequence CAGGAT is also found 64 nucleotides upstream. It has been pointed out that some eukaryotic promoters have a sequence similar to GG (CT) CAATCT approximately 40 base pairs upstream from the TATAAAA sequence (Chang *et al.* 1998). But there is not the sequence in *Rhinopithecus roxellanae* growth hormone gene.

In corresponding introns, the pattern of divergence involves two changes in size (due to deletions and insertions) and base substitutions. Introns evolve much more rapidly than exons, in comparisons of the same gene in different species, sometimes the exons are homologous, while the introns have diverged so much that corresponding sequences cannot be recognized.

Mutations occur at the same rate in both exons and introns, but are removed more effectively from the exons by adverse selection. However, in the absence of the constraints imposed by a coding function, an intron is able quite freely to accumulate point substitutions and other changes. These changes imply that the intron does not have a sequence-specific function. Whether its presence is at all necessary for gene function is not clear.

There are multiple growth hormone genes in the human genome (Vize *et al.* 1987). A comparable multiple gene structure does not appear to be found for the growth hormone gene in *Rhinopithecus roxellanae.*

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R. roxellanae	GAGGAGGTTC	TAAATTATCC	ATCAGCACAG	GCCGCCCAGT	GGCCCGATGC	ATAAATGTAT
Porcine	GAGGAGGTTG	TAAATTATCC	ATTAGCACAT	GCCTGCCAGT	GGGCC :ATGC	ATAAATGTAT
#1	GAGGAGGTTS	TAAATTATCC	ATYAGCACAK	GCCKSCCAGT	GGSCCGATGC	ATAAATGTAT
R. roxellanae	AGAGAAAATA	AGTGGGGGCG	GAGGGGGATA	GAGAAGGGAC	CAGGGTATAA	AAAGGGCCTG
Porcine	AGAGAAAATA	GGTGGGGGCA	GAGGGAGA: :	GAGAAGAGGC	CAGGGTATAA	AAAGGGCCCA
# 61	AGAGAAAATA	RGTGGGGGCR	GAGGGRGATA	GAGAAGRGRC	CAGGGTATAA	AAAGGGCCYR
R. roxellanae	CAAGGGACTG	ATTCCAGGAT	CCCAGCACCC	GGCTCCCCAA	ACCGCTCAGG	GTCCTGTGGA
Porcine	AAAGGGACCA	ATTCCAGAAT	CCCAGGACCC	AGCTCCCCAG	ACCACTCAGG	GACCTGTGGA
#121	MAAGGGACYR	ATTCCAGRAT	CCCAGSACCC	RGCTCCCCAR	ACCRCTCAGG	GWCCTGTGGA
R. roxellanae	CGGCTCATCC	CCAGCTGTGa	tggctgcagG	TAAGTGCCCC	TAAAATCCCT	TTGGGTTCAG
Porcine	CAGCTCA :CC	G : :GCTGTGa	tggctgcagG	CAAGTGCCCC	TAAAATCCCA	GTGGCTTG :G
#181	CRGCTCATCC	SCAGCTGTGa	tggctgcagG	YAAGTGCCCC	TAAAATCCCW	KTGGSTTSAG
R. roxellanae	TGTATACTGA	GGCACAAGGC	GGTGGCCCGG	CATGTGGATG	GGGATACTAA	CCCTGGGCTT
Porcine	TGTCTTCTGA	AGGGTGACGT	GGGGGCCATG	CAGATGGATG	GGGC :ACCAA	CCTTGGGCTT
#241	TGTMTWCTGA	RGSRYRASGY	GGKGGCCMKG	CAKRTGGATG	GGGMTACYAA	CCYTGGGCTT
R .roxellanae	TGGGGCTGCT	GAATGTGAGC	AGAGACATCT	ATGCCCAGAC	ATTTGGCCAA	GTTTTAAATG
Porcine	TGGGGTTTCC	GAATGTGAGC	ATGGATATCT	ACTCCTAGAT	ATGAGGCCAA	GTTTTAAATG
#301	TGGGGYTKCY	GAATGTGAGC	AKRGAYATCT	AYKCCYAGAY	ATKWGGCCAA	GTTTTAAATG
R .roxellanae	TTC :TCCGTC	CCCGGGNGGG	GAGGGGA:AC	GGCTGACGGG	AAACAGAGGC	CTCTTGCTGT
Porcine	TCCCTGGGGG	AGGGGGAGGA	GAAGGGACAG	GGCTGGTGG:	AGCCAG :G :C	CTCTTG :TCT
#361	TYCCTSSGKS	MSSGGGAGGR	GARGGGACAS	GGCTGRYGGG	ARMCAGAGGC	CTCTTGCTST
R. roxellanae	CTGGGCCACG	GCCTCGCCCT	CCGGGTCCCT	C : T : : : CTGT	AGgccctcgg	aactctgtgc
Porcine	CTGGGAT :C :	:CCTCTCTCA	C GGG :CCCT	CCTGGTCTCT	AGgccctcgg	acctccgcgc
#421	CTGGGMYACG	GCCTCKCYCW	CCGGGTCCCT	CCTGGTCTST	AGgccctcgg	amctcygygc
R. roxellanae	Tcctggcgtt	tgccctgctc	tgcctgccct	Ggccccagga	ggtgggcgcc	ttcccagcca
Porcine	Tcctggcttt	cgccctgctc	tgcctgccct	Ggactcggga	ggtgggcgcc	ttcccagcca
#481	Tcctggcktt	ygccctgctc	tgcctgccct	Ggmcycrga	ggtgggcgcc	ttcccagcca
R.roxellanae	Tgcccttgtc	cagtctgttt	gccaacgccg	Tgctccgggc	ccagcacctg	c
Porcine	Tgcccttgtc	cagcctattt	gccaacgccg	Tgctccgggc	ccagcacctg	caccaactgg
#541	Tgcccttgtc	cagyctrttt	gccaacgccg	Tgctccgggc	ccagcacctg	caccaactgg

Fig.3. The partial sequences growth hormone gene are compared between *Rhinopithecus roxellanae* and porcine

S: Substitution between C and G; Y: Substitution between C and T; K: Substitution G and T; R: Substitution between A and G; M: substitution between C and A; W: Substitution between T and A; **:** incorresponding nucleotide; The exon is indicated by small letter.